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(54) Title: FLUOROGENIC PROTEASE SUBSTRATES

(57) **Abstract:** Fluorogenic protease substrates are provided that comprise a peptide doubly labelled via thiol groups of the peptide with an alkyleneamidotetramethylrhodamine (alkyleneamido-TMR) group. Preferred substrates are doubly labelled with substantially pure 5-methyleneamido-TMR or 6-methyleneamido-TMR. Methods of preparing the substrates are provided, which comprise reacting the unlabelled peptide with haloalkylamido-TMR (preferably iodoacetamido-TMR). More generally, fluorogenic protease substrates are also provided which comprise a peptide doubly labelled with the same rhodamine derivative, where the two labels, and their linkages to the peptide, are substantially isomerically identical. Also provided are related methods for assaying protease activity in a sample, kits for use in such methods, and solid supports bearing the substrates of the invention.